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ABSTRACT

Hydroponic culture solutions containing bicarbonate (HCO_3^-) may be used to screen crops such as soybeans (*Glycine max*) for resistance to iron (Fe) deficiency or chlorosis. Some successful methods use sodium bicarbonate ($NaHCO_3$) in combination with elevated partial pressures of carbon dioxide (CO_2) to buffer pH and elevate bicarbonate. Replacing $NaHCO_3$ with magnesium bicarbonate [$Mg(HCO_3)_2$] as the form of bicarbonate alkalinity has the potential to produce culture solutions that simulate soil solutions more closely and eliminate any potential for specific sodium (Na) toxicities in sensitive plants. A modified screening solution based on $Mg(HCO_3)_2$-CO_2 was tested against the successful $NaHCO_3$-CO_2 method, using three soybean varieties of known resistance to Fe-deficiency chlorosis. Alkalinity was 10 mM [added as $NaHCO_3$ or $Mg(HCO_3)_2$], solutions were aerated with 3% CO_2, and Fe was provided as FeDTPA (diethylenetriamine-pentaacetic acid) at 15 μM (low Fe) or 60 μM (adequate Fe). Leaf chlorophyll, visual chlorosis index, and leaf Fe concentration were closely related. Solutions based on $NaHCO_3$ or $Mg(HCO_3)_2$ provided identical chlorosis-susceptibility rankings for the three cultivars.

Keywords: Bicarbonate-induced chlorosis, iron deficiency, plant nutrition, chlorosis screening, bicarbonate, sodium, magnesium, nutrient solution, soybean, *Glycine max*

INTRODUCTION

Iron (Fe) chlorosis is a problem for soybeans (*Glycine max*) and other crops grown in calcareous soils. Efforts to improve resistance to chlorosis by plant

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breeding are enhanced by simple and reliable screening methods to assess the resistance of parent genotypes and their progeny. Screening for chlorosis resistance in the field is time consuming and has been inconsistently reliable with soybean genotypes, giving variable results influenced by soil heterogeneity, environmental conditions, and seasonal trends (Fairbanks, 2000). Screening in potted soils has sometimes been more successful than in the field, providing that water availability, bulk density, and soil chemical conditions were carefully chosen. However, many researchers have chosen to screen for chlorosis resistance in nutrient culture solutions because of their convenience, reproducibility, and the ease with which chemical conditions can be manipulated.

Soluble bicarbonate has long been recognized as a contributor to Fe deficiencies and lime-induced chlorosis of crops growing on calcareous soils (e.g., Harley and Lindner, 1945; Wadleigh and Brown, 1952; Brown et al., 1959; Boxma, 1972; Coulombe et al., 1984a). Although the role of bicarbonate is not fully understood, it is thought to inhibit the uptake or utilization of Fe by plants, as well as to buffer the pH in an alkaline range (Chaney et al., 1992; Parker and Norvell, 1999; Brand et al., 2000; Lucena, 2000). Soluble bicarbonates have been added to a wide variety of culture solutions designed to induce Fe deficiency or chlorosis in plants (Coulombe et al., 1984b; Parker and Norvell, 1999). Sodium bicarbonate has been the most common source of bicarbonate added to culture solutions, but $KHCO_3$ has also been used. Additions of bicarbonate have sometimes been accompanied by solid-phase $CaCO_3$, but this addition seems undesirable because a potentially adsorptive and sparingly soluble solid is introduced, which may cause variability in solution composition.

Concentrations of bicarbonate added to plant culture solutions have ranged widely, from less than 1 mM to at least 50 mM (e.g., Coulombe et al., 1984b; Campbell and Nishio, 2000; Porter and Thorne, 1955; Shi et al. 1993; Viti and Cinelli, 1993). Concentrations of bicarbonate in the low mM range provide relatively ineffective control of pH. Higher concentrations of bicarbonate can provide more stable pH values, but the use of high bicarbonate alkalinity in the absence of elevated CO_2 tends to raise the pH of solutions above 8, and permits further upward drift in pH as CO_2 is lost. High pH values are undesirable in plant culture solutions because they restrict the solubility or availability of several nutrients besides Fe, e.g., calcium (Ca), copper (Cu), phosphorus (P), and zinc (Zn) (Parker and Norvell, 1999; Norvell, 1991). In addition, high pH values are not necessarily representative of calcareous soils, where equilibria among Ca^{2+}, HCO_3^-, $CaCO_3$, and elevated partial pressures of CO_2 usually establish more moderate pH values (Bloom, 2000). The latter are typical of many calcareous soils under somewhat wet conditions, which seem to induce or exacerbate chlorosis of soybeans (Inskeep and Bloom, 1984).

Combining added bicarbonate with controlled partial pressures of CO_2 provides a means to regulate the pH of plant culture solutions at reasonable values with a good buffering capacity (Chaney et al., 1992; Porter and Thorne,

1955; Brown et al., 1959; Parker and Norvell, 1999). After several years of experimenting with CO_2-enriched aeration of bicarbonate-buffered nutrient solutions, Chaney et al. (1989, 1992) and Coulombe et al. (1984b) used this approach to develop improved methods to screen soybeans for chlorosis resistance. In addition to adding $NaHCO_3$ and aerating with CO_2-enriched air to regulate pH, they raised concentrations of Ca^{2+} and magnesium (Mg^{2+}) to better represent the soil solution of calcareous soils, and they used chelator buffering to regulate the availability of Fe and other micronutrient metals. These improvements have provided increasingly reliable and convenient screening techniques for soybeans (Chaney et al., 1992; Fairbanks, 2000).

The data of Inskeep and Bloom (1984) for soil solutions suggest that the culture solutions developed by Chaney et al. (1992) could be modified somewhat further to even more closely simulate the soil solution of wet calcareous soils where chlorosis of soybeans is often observed. Adding bicarbonate as $Mg(HCO_3)_2$ rather than as $NaHCO_3$ would provide HCO_3^- without raising the concentration of Na^+, and simultaneously this change would provide elevated Mg^{2+} without the need to raise anions other than HCO_3^-. In addition to more closely matching the composition of soil solutions of many calcareous soils of the midwestern US, the elimination of $NaHCO_3^-$ avoids any potential for the accumulation or toxicity of Na in sensitive crops (Tester and Davenport, 2003).

Our first objective in this study was to improve methods for buffering the pH of plant culture solutions by using $Mg(HCO_3)_2$ as the source of alkalinity in solutions using HCO_3^- in combination with CO_2-enriched aeration. While conceptually simple, this goal necessitated finding a convenient means to bring adequate concentrations of $Mg(HCO_3)_2$ into solution, because $Mg(HCO_3)_2$ itself is not available as a stable solid, and $MgCO_3$ is too sparingly soluble to provide convenient stock solutions. Our second objective was to compare a chlorosis-screening solution based on $Mg(HCO_3)_2$-CO_2 with an otherwise similar solution based on $NaHCO_3$-CO_2 in terms of their ability to buffer pH and discriminate successfully among soybean genotypes differing in chlorosis susceptibility. For the latter purpose, three genotypes known to differ in susceptibility to chlorosis in the field were selected (Chaney et al., 1992): Wayne (highly susceptible), Williams (moderately susceptible), and A7 (moderately resistant).

MATERIALS AND METHODS

Soybean Culture

Approximately 75 seeds of cultivars Wayne, Williams, and A7 were treated with Thiram (Tetramethylthiuram disulfide) at a rate of about 20 μL of 42% concentrate per seed, then air-dried. Seeds were imbibed for 1 d in aerated

solutions of 0.5 mM $CaSO_4$ and 10 μM H_3BO_4, then germinated on moist filter paper. On day 3 after imbibition, the elongating radicles of germinating seedlings were inserted through the screened bases of black plastic cups. These plant cups were inserted into holes in the lids of 5 L, black polyethylene buckets so that the seedling radicles were suspended in aerated nutrient culture solution containing macroelements plus boron (B) and molybdenum (Mo) (solution described below). Each combination of cultivars (three), buffers (two), and Fe treatments (two) was replicated twice for a total of 24 pots. Four or five seedlings per pot of each cultivar were planted initially, but these were thinned to two relatively uniform plants per pot prior to imposition of treatments. As the soybean shoots extended, black polyethylene beads were added to cups to support the plants and to exclude light from culture solutions.

Mixed fluorescent and incandescent lights in the growth chamber were turned on at 25% of full intensity on day 5, at 50% of full intensity on day 6, and at full intensity after day 6 (full intensity was approximately 580 $\mu E/m^2s$). The day cycle was 16 h at 24°C, and the night cycle was 8 h at 21°C.

Aeration of pots with air enriched in CO_2 was started on day 7, when bicarbonate was added as either $NaCO_3$ or $Mg(HCO_3)_2$ as described below. Diethylenetriaminepentaacetic acid (DTPA) chelates of Zn, Cu, manganese (Mn), nickel (Ni), and cobolt (Co) were added on day 8, and an excess of DTPA was included to buffer metal-ion activities. Iron was included at either 15 μM FeDTPA (intended to cause Fe deficiency) or 60 μM FeDTPA (intended to provide adequate or near-adequate amounts of Fe). Small amounts of P and B were added every day or two after day 10 to replace these nutrients at a rate similar to their removal by plant uptake.

Chlorosis was rated visually on day 22, using a six-point scale for a visual chlorosis index (VCI) similar to that of Chaney et al. (1992): 0 = fully green; 1 = pale green or mottled green with little or no differentiation between veins and interveinal areas; 2 = mildly chlorotic with yellowish-green interveinal areas and green veins; 3 = fully chlorotic with mostly yellow interveinal areas and green veins; 4 = severely chlorotic with yellow interveinal areas, occasional necrotic speckling, and pale green or yellow veins; 5 = very severe chlorosis with yellow to white interveinal areas, common necrotic speckling, death of growing point, and severely chlorotic side shoots (if present).

On day 23 (15 d after imposing treatments), the first, second, and third trifoliolate leaves (T1, T2, and T3) were harvested from the four plants in each treatment. A pair of disks, 2.15 cm in diameter, were cut from the middle of the center leaflet of each trifoliolate leaf. One was extracted by dimethylformamide for spectrophotometric analysis of chlorophyll (Inskeep and Bloom, 1985), and the other dried at 65°C and weighed. The remainder of the leaf was dried, weighed, ashed at 525°C, dissolved in concentrated HNO_3, and analyzed for Fe by inductively coupled argon plasma emission spectrometry.

Nutrient Solutions and pH Buffering

All culture solutions were prepared to contain the following macroelements (mM): KNO_3, 2.5; $Ca(NO_3)_2$, 5.0; $MgSO_4$, 2.0; NaCl, 0.5; KH_2PO_4, 0.02; and microelements (μM): H_3BO_4, 10; and MoO_3, 0.2. In addition, a supplement equivalent to 10 μM P and 0.1 μM B was added every day or two after day 10 at a rate intended to be adequate for growth. On day 8, all pots received (μM): ZnDTPA, 24; CuDTPA, 12; MnDTPA, 10; NiDTPA, 1; and CoDTPA, 1; plus an additional 100 μM of excess DTPA. Finally, two levels of Fe were added: 15 μM FeDTPA was the low Fe-treatment intended to cause Fe-deficiency stress, and for comparison, 60 μM FeDTPA was added to provide adequate or near-adequate levels of available Fe. Reagent-grade chemicals and double-deionized water were used to prepare all solutions.

Bicarbonate at a concentration of 10 mM was included as a pH buffer in combination with aeration by 3% CO_2-enriched air, as recommended by Chaney et al. (1992) for chlorosis-susceptibility screening of soybeans. This combination of HCO_3^- and CO_2 was intended to provide a pH of approximately 7.5. Higher or lower pH values can easily be obtained by changing the concentration of bicarbonate alkalinity or the concentration of CO_2 according to well-recognized chemical principles (Chaney et al., 1992; Parker and Norvell, 1999). Culture solutions buffered by $NaHCO_3$ were prepared by adding 10 mL of 1 *M* stock per liter of culture solution. The solutions buffered by $Mg(HCO_3)_2$ were prepared by adding 40 mL of 0.125 *M* stock per L of culture solution. Because of the limited solubility of $MgCO_3$, the stock solution of $Mg(HCO_3)_2$ was prepared by adding 5.04 g of reagent-grade MgO per liter of H_2O, followed by continuous mixing of the MgO suspension by bubbling with 100% CO_2. This mixing was continued for 2–3 h until dissolution was complete and the pH had decreased to between 6.9 and 7.0. Stock solutions of $Mg(HCO_3)_2$ prepared in this manner remained stable for at least several weeks, as long as they were tightly closed to prevent loss of CO_2.

All pots containing culture solutions were aerated at a rate of about 250 cc/min with air containing CO_2 at a concentration of approximately 3%. The aeration gas was formed by combining the independently regulated flows of air and CO_2. The air supply was filtered-compressed air with flow controlled by an Air Products flow regulator (E29R-150MM4SS). The CO_2 source was a tank of compressed CO_2 with flow controlled by a two-stage pressure regulator and an Air Products flow meter (E29C-150MM2SS).[1] The CO_2-enriched air was passed through a 0.45 μm cartridge filter before distribution to pots through FDA food-grade polyvinyl tubing. The aeration rate was equalized among pots by capillary restriction of flow from the tubing of the main supply line.

[1]Mention of trademark, proprietary product or vendor does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

Statistical analyses were conducted using SAS (Statistical Analysis System, SAS Institute, Cary, NC). The chlorophyll and Fe results were analyzed using PROC GLM. Because of the inherently ordinal nature of the scale for visual estimates of chlorosis, the VCI results were analyzed using SAS PROC MIXED using the ANOVA-like nonparametric approach described in detail by Shah and Madden (2004). The estimated relative treatment effects for VCI were computed for each treatment using rank-transformed data (computations not presented).

RESULTS AND DISCUSSION

Effectiveness of pH Buffering

Bicarbonate added as $Mg(HCO_3)_2$ or $NaHCO_3$ at 10 mM provided similar and effective control of pH in culture solutions aerated with 3% CO_2. No significant differences in pH were observed between treatments differing in bicarbonate source, nor among treatments differing in the soybean cultivar used. Solution pH values were affected slightly by soybean responses to Fe treatments. On Day 23, 15 d after imposition of treatments, solutions with low Fe (15 μM FeDTPA) averaged 7.60 ± 0.02 in pH, whereas in solutions with adequate Fe (60 μM FeDTPA), the pH averaged 7.67 ± 0.04. Overall, solution pH values varied from about 7.4 to 7.7 during the course of the experiment (data not shown), but the main cause of this variability was minor fluctuations in the flow rates for CO_2 or air, which often caused day-to-day variations of about 0.1 pH units.

Soybean Growth and Appearance

All plants had developed three trifoliolate leaves by harvest on day 23. The first and second trifoliolate leaves, T1 and T2, were fully expanded or nearly so. The third trifoliolate leaf, T3, was not fully expanded on most plants, especially under the low-Fe treatment at 15 μM FeDTPA. The T3 leaf was particularly small on Fe-stressed plants of Wayne, the cultivar most susceptible to chlorosis in the field. At the 60 μM level of FeDTPA, all cultivars grew well and their leaves appeared fully green in both buffer systems. At the 15 μM level of FeDTPA in both buffer systems, the leaves of all cultivars were smaller and most displayed moderate to severe chlorosis. The most severely chlorotic leaves were nearly white, with small necrotic speckles.

Chlorophyll, Visual Chlorosis Index, and Iron Concentration of Leaves

The concentrations of chlorophyll and Fe in soybean leaves, as well as our visual assessments of chlorosis, were not influenced by bicarbonate source,

Table 1
Analysis of variance for chlorophyll, visual chlorosis index (VCI), and Fe in the second trifoliolate leaf of three soybean cultivars

	Soybean Leaf Characteristics		
Variables	Chlorophyll	VCI	Fe
Cultivar	***	***	***
Buffer	ns	ns	ns
Fe	***	***	***
Cultivar × Fe	*	*	ns
Cultivar × Buffer	ns	*	ns
Fe × Buffer	ns	ns	ns
Cultivar × Buffer × Fe	ns	*	ns

***, **, * $P \leq 0.005$, 0.01, and 0.05 for main effects.
* $P < 0.2$ for interactions.
ns = Not significant.

Table 2
Chlorophyll (ug cm^{-2})* in trifoliolate leaves of three soybean cultivars grown with low or adequate Fe supply in nutrient solutions containing $NaHCO_3$ or $Mg(HCO_3)_2$

	Low Fe		Adequate Fe	
Genotype	$NaHCO_3$	$Mg(HCO_3)_2$	$NaHCO_3$	$Mg(HCO_3)_2$
		Trifoliolate Leaf 1		
A7	33.0	33.9	52.7	52.6
Williams	16.5	17.5	49.4	52.2
Wayne	7.4	5.5	44.5	41.0
LSD	5.6	5.6	4.1	4.8
		Trifoliolate Leaf 2		
A7	26.0	28.1	51.8	50.4
Williams	22.7	17.5	48.2	49.8
Wayne	14.3	10.7	47.4	45.6
LSD	10.7	4.5	3.5	3.8
		Trifoliolate Leaf 3		
A7	21.0	25.9	47.7	45.1
Williams	23.0	13.7	38.4	38.4
Wayne	13.2	10.4	37.8	40.7
LSD	6.5	11.1	5.4	3.9

*Mean total chlorophyll in trifoliolate leaves of four plants of a cultivar within an Fe × buffer treatment. LSD is given at $p = 0.05$ level for each leaf for comparison of cultivars within a treatment. There were no significant differences between buffers within an Fe treatment for any genotype (see Table 1).

but were strongly influenced by cultivar and Fe supply. These relationships are shown by the ANOVA in Table 1 for T2, the youngest fully expanded leaf, which is often used as an indicator of nutritional status in soybeans and other crops (Reuter, 1986). Concentrations of chlorophyll in T1, T2, and T3 were generally similar (Table 2), even though the characteristics of T1 are potentially more influenced by seed Fe, and leaf T3 was still quite immature on some plants.

Screening solutions based on either $Mg(HCO_3)_2$ or $NaHCO_3$ were generally successful in ranking the soybean cultivars by leaf chlorophyll (Table 2) in the same order of chlorosis resistance observed in the field (i.e., A7 > Williams > Wayne). The rankings were more consistent and more often significant for T1 and T2 than for the more immature T3. Although differences between bicarbonate sources were not significant, there was a tendency toward larger,

Table 3
Visual chlorosis index* of trifoliolate leaves of three soybean cultivars grown with low or adequate Fe supply in nutrient solutions containing $NaHCO_3$ or $Mg(HCO_3)_2$

	Low Fe		Adequate Fe	
Genotype	$NaHCO_3$	$Mg(HCO_3)_2$	$NaHCO_3$	$Mg(HCO_3)_2$
		Trifoliolate Leaf 1		
A7	1.2 (0.5) a	1.0 (0.0) d	0.0 (0.0) g	0.0 (0.0) j
Williams	3.5 (0.1) b	2.7 (0.6) e	0.0 (0.0) g	0.0 (0.0) j
Wayne	4.5 (0.0) c	4.5 (0.1) f	0.0 (0.0) g	0.5 (1.0) k
		Trifoliolate Leaf 2		
A7	1.2 (0.6) a	1.0 (0.0) d	0.0 (0.0) g	0.0 (0.0) j
Williams	2.5 (0.0) b	2.5 (0.1) e	0.0 (0.0) g	0.0 (0.0) j
Wayne	3.0 (1.0) b	3.7 (0.5) f	0.0 (0.0) g	0.0 (0.0) j
		Trifoliolate Leaf 3		
A7	1.5 (1.1) a	1.2 (0.5) d	0.0 (0.0) g	0.0 (0.0) j
Williams	1.8 (0.6) a	2.5 (0.1) e	0.0 (0.0) g	0.0 (0.0) j
Wayne	3.0 (1.0) b	3.5 (0.0) f	0.0 (0.2) g	0.5 (1.0) j

*Median and (interquartile range) for visual chlorosis index (0–5 scale; see Materials and Methods) for the trifoliolate leaves of the four plants in each buffer × Fe treatment. For comparison of cultivars within an Fe × buffer treatment, the VCI values for a trifoliolate leaf followed by the same letter within a column are not significantly different based on relative treatment effects estimated by the method of Shah and Madden (2004). There were no significant pair-wise differences between buffers within an Fe treatment for any genotype (see Table 1).

and therefore more easily measured, differences in chlorophyll among cultivars when grown in the $Mg(HCO_3)_2$-buffered solutions.

Visual chlorosis index values were estimated for all trifoliolate leaves (Table 3). Although less precise and more subjective than chemical analyses, the VCI estimates were, in fact, well correlated with leaf chlorophyll (Figure 1). Rankings of cultivars for chlorosis resistance based on VCI were similar to those based on total chlorophyll in leaves.

The Fe concentration of leaves also differed among cultivars (Table 4), but fewer differences were significant. The chlorosis-resistant cultivar, A7, generally contained the highest concentration of Fe in leaves of plants from any given treatment. In the low-Fe treatment, A7 accumulated roughly 50% more leaf Fe than the least-resistant cultivar, Wayne. Differences in Fe concentrations between leaves of Williams and Wayne were smaller and often insignificant. Concentrations of Fe (in milligrams per kilogram) and chlorophyll (in grams

Table 4
Mean concentration of Fe (ug g^{-1})* in trifoliolate leaves of three soybean cultivars grown with low or adequate Fe supply in nutrient solutions containing $NaHCO_3$ or $Mg(HCO_3)_2$

	Low Fe		Adequate Fe	
Genotype	$NaHCO_3$	$Mg(HCO_3)_2$	$NaHCO_3$	$Mg(HCO_3)_2$
		Trifoliolate Leaf 1		
A7	38	51	71	81
Williams	31	31	71	66
Wayne	26	24	66	61
LSD	12	5	18	10
		Trifoliolate Leaf 2		
A7	48	52	84	86
Williams	48	32	72	64
Wayne	30	27	67	68
LSD	27	10	7	8
		Trifoliolate Leaf 3		
A7	46	55	78	79
Williams	47	32	62	59
Wayne	33	31	63	68
LSD	23	14	10	7

*Mean total Fe in trifoliolate leaves of four plants of a cultivar within an Fe × buffer treatment. LSD is given at $p = 0.05$ level for each leaf for comparison of cultivars within a treatment. There were no significant differences between buffers within an Fe treatment for any genotype (see Table 1).

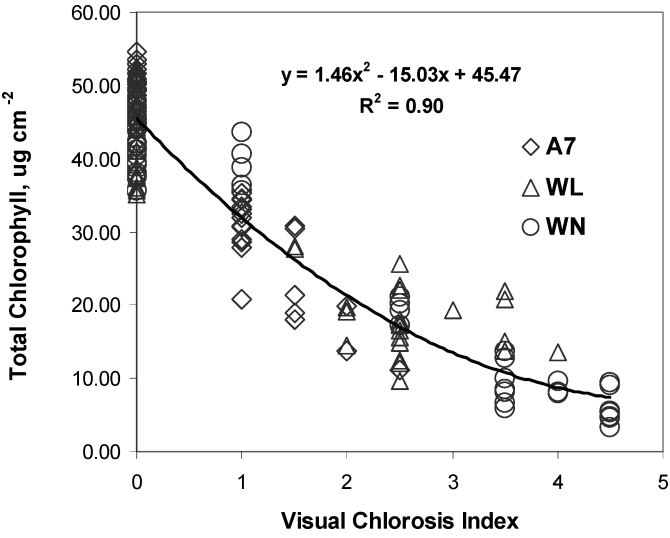

Figure 1. Relationship between total chlorophyll (ug cm^{-2}) and visual chlorosis index (0–5 scale, see Methods) for trifoliate leaves 1, 2, and 3 of soybean cultivars A7, Williams, and Wayne.

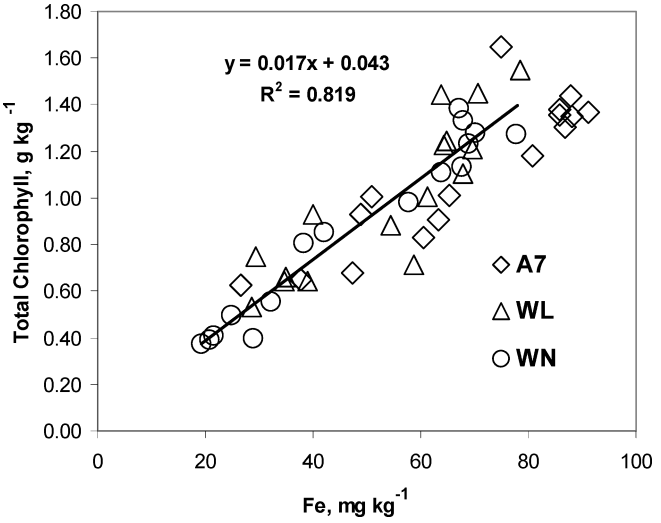

Figure 2. Relationship between total chlorophyll (g kg^{-1}) and Fe concentration (mg kg^{-1}) in trifoliate leaf 2, the youngest fully expanded leaf from the three soybean cultivars A7, Williams, and Wayne. The regression line covers the range of data below 75 mg kg^{-1} Fe.

per kilogram) in leaves were closely related, as shown in Figure 2 for T2, the youngest, most fully developed leaf on each plant. A reasonably close relationship between these characteristics was expected because chlorophyll development was intentionally limited by restricting Fe availability. Although too few Fe treatments were included to define adequately a critical concentration of leaf Fe, the results suggest that about 65 to 75 mg kg^{-1} of leaf Fe were needed to achieve maximum chlorophyll development in the three cultivars. This estimate is somewhat higher than the concentration of about 50 mg kg^{-1} Fe (Adams et al., 2000; Brown and Jones, 1977) that has been suggested to be minimally adequate for certain other soybean cultivars.

CONCLUSIONS

Bicarbonate supplied as $Mg(HCO_3)_2$ or $NaHCO_3$ was effective as a pH buffer in plant culture solutions which were aerated with CO_2-enriched air. A concentration of 10 mM HCO_3^- aerated with approximately 3% CO_2 in air maintained a pH of about 7.6. These concentrations of HCO_3^- and CO_2 were chosen to approximate the conditions recommended by Chaney et al. (1992), but higher or lower pH values can easily be obtained by changing the concentration of bicarbonate alkalinity or the concentration of CO_2 according to well-recognized chemical principles.

Plant culture solutions using either $Mg(HCO_3)_2$ or $NaHCO_3$ were successful in correctly ranking soybean cultivars known to differ in susceptibility to chlorosis in the field. Differences in chlorophyll, visual chlorosis index, or Fe concentration of soybean leaves were strongly influenced by Fe supply and cultivar, but not by bicarbonate source. The results suggest that $Mg(HCO_3)_2$ can be readily substituted for $NaHCO_3$ in chlorosis-screening solutions or in other bicarbonate-buffered plant culture solutions to provide a solution environment that is closer in composition to the soil solutions of calcareous soils.

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